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(54) Title: GLYCOCONJUGATE INHIBITORS OF HUMAN SPERM-EGG BINDING

(57) Abstract

A human glycoprotein can be used as a contraceptive agent. The oligosaccharides of the glycoprotein can also be used. The oligosaccharides can be conjugated to other polymers and substances for use in diagnostic and therapeutic methods regarding infertility.

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GLYCOCONJUGATE INHIBITORS OF HUMAN SPERM-EGG BINDING

TECHNICAL FIELD OF THE INVENTION

This invention relates to glycoconjugate inhibitors of human sperm-egg binding and their use as diagnostic and prognostic indicators for human fertilization. The invention relates specifically to those carbohydrate sequences that block sperm-zona pellucida binding.

BACKGROUND OF THE INVENTION

Complex carbohydrates have been shown to play essential roles in several fundamental recognition processes in mammalian cells. Recent data indicate that the initial binding of leukocytes and platelets to vascular endothelium during inflammation is mediated by lectin-like interactions between surface carbohydrates and complementary adhesive glycoproteins known as selectins (VARKI, 1994). The appropriate recognition of surface carbohydrates is also a crucial event in the binding of sperm to eggs in diverse species from both the plant and animal kingdoms. Mammalian sperm-egg binding is no exception to this rule based on studies carried out in several model systems (WASSARMAN, 1990). The egg binding protein on mouse and hamster sperm have been shown to initially bind carbohydrate sequences associated with a specific protein (designated ZP3) present on the zona pellucida, the specialized extracellular matrix surrounding the mammalian egg (WASSARMAN, 1990). The specific carbohydrate sequence that mediates human sperm-zona pellucida binding has not been determined due to a lack of sufficient material for structural and functional analysis. A human analogue of the mouse ZP3 glycoprotein has been identified and cloned (CHAMBERLIN, 1990), but no recombinant form has been shown to inhibit

human sperm-zona pellucida binding possibly because of the lack of expression of the appropriate carbohydrate sequence in recombinant cell lines (CHAMBERLIN, 1990).

Human sperm manifest a very high degree of specificity for binding to their homologous eggs, unlike sperm from the lower mammalian species (BEDFORD, 1977). Human sperm will only bind to eggs from other higher primates (chimpanzees, gibbons, orangutans, and gorillas). However, because of very highly restricted access to such endangered higher primates, data about human sperm-zona pellucida binding is often obtained using an *in vitro* system utilizing human sperm and human eggs known as the hemizona assay (HZA) (BURKMAN, 1988). The HZA involves the microbisection of the human egg, resulting in the release of the egg cell contents and the generation of two equally matched hemispheres of the zona pellucida (hemizonae). By comparing the binding of fertile sperm in the presence and absence of a test substance, it is possible to quantitate the contraceptive effect of the test substance using this internally controlled assay system. The HZA has been used previously to test a number of different oligosaccharides, polysaccharides and glycoproteins for their ability to inhibit human sperm-egg binding (OEHNINGER, 1990, 1991). However, no substances have been found which are endogenous and which are effective at very low concentrations to inhibit sperm-egg binding. Thus there is a need in the art for such substances to enlarge the contraceptive options available to humans.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for inhibiting human sperm-zona pellucida binding.

It is another object of the invention to provide contraceptive methods for use in humans.

It is yet another object of the invention to provide a method for assessing human sperm dysfunction.

It is still another object of the invention to provide a method for determining a cause of infertility.

It is an object of the invention to provide a contraceptive composition.

It is still another object of the invention to provide a contraceptive device.

It is an object of the invention to provide a method of screening for contaceptive substances.

It is another object of the invention to provide a method of screening for autoantibodies which contribute to infertility.

It is yet another object of the invention to provide a method of treating an individual aberrantly expressing endometrial PAEP-associated oligosaccharides. These and other objects of the invention are provided by one or more embodiments as described below.

According to one embodiment of the invention a method is provided for inhibiting sperm-zona pellucida binding. The method comprises:

administering a composition comprising endometrial PAEP-associated oligosaccharides to a female, in an amount effective to inhibit human sperm-zona pellucida binding.

According to another embodiment of the invention a second method for inhibiting human sperm-zona pellucida binding is provided. The method comprises:

administering to a female a biological macromolecule which specifically binds to endometrial PAEP.

In another embodiment of the invention a diagnostic method is provided for assessing human sperm dysfunction. The method comprises:

determining the number of binding sites for endometrial PAEP on a sample of human sperm, wherein said number of binding sites correlates with ability of said sperm to bind to human zona pellucida.

In still another embodiment of the invention a method for assessing a cause of infertility is provided. The method comprises:

detecting expression of oligosaccharides normally found on endometrial PAEP in secretions or tissues of a male or female reproductive tract

wherein said oligosaccharides are not expressed in said secretions or tissues of fertile humans.

According to yet another embodiment of the invention a method of treating an individual aberrantly expressing endometrial PAEP-associated oligosaccharides, is provided. The method comprises:

administering a biological macromolecule which specifically binds endometrial-associated oligosaccharides to an individual aberrantly expressing endometrial PAEP-associated oligosaccharides, wherein said biological macromolecule is administered in an amount sufficient to promote human sperm binding to human zona pellucida.

In still another embodiment a contraceptive composition is provided. The composition comprises an endometrial PAEP-associated oligosaccharide and a pharmaceutically acceptable carrier for vaginal, or intrauterine administration.

In another embodiment of the invention a contraceptive device is provided which is impregnated with a composition comprising an endometrial PAEP-associated oligosaccharide and a pharmaceutically acceptable carrier, wherein said device is selected from the group consisting of: condoms, intrauterine devices, diaphragms, and sponges.

According to another embodiment of the invention a method of screening for contraceptive substances is provided. The method comprises:

contacting a test substance with endometrial PAEP-associated oligosaccharides; and

determining those test substances which bind to endometrial PAEP-associated oligosaccharides.

In another embodiment of the invention a method of screening for contraceptive substances is provided. The method comprises:

contacting a test substance with endometrial PAEP-associated oligosaccharides; and

determining the presence of antibodies in the body sample which specifically bind to endometrial PAEP-associated oligosaccharides.

Thus the present invention provides the art with methods, compositions, and devices for safely and effectively blocking conception in humans.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 schematically represents the concentration dependent inhibition of endometrial PAEP derived from human amniotic fluid on human sperm-zona pellucida binding in the hemizona assay system. Concentration is expressed as μg/ml on the X-axis. The Y axis is the hemizona index (HZI). The HZI is calculated on the basis of the following formula: the (number of sperm bound in the presence of a test substance/number of sperm bound in the absence of the test substance) X 100. Therefore the HZI is a percentage of normal binding of sperm in the presence in this case of endometrial PAEP. It is notable that 50% inhibition of sperm binding is observed at a final concentration between 1 and 10 μg/ml, providing an IC50 value between 3.6 X 10-8 M and 3.6 X 10-7 M assuming a molecular weight of 28,000 Da (28 kDa). Complete inhibition of binding is observed at a concentration not exceeding 50 μg/ml (1.8 X 10-6 M). The inhibitory dose dependence analyzed by the ANOVA method of statistics has a p value < 0.0001, with the number of hemizona employed for each point ranging from 4-9.

Fig. 2 is the established protein sequence of endometrial PAEP using conventional abbreviations for amino acid (JULKUNEN, 1988) indicating at the arrow the sites of attachment of the oligosaccharides of endometrial PAEP (asparagine at amino acid 28 and amino acid 63).

Fig. 3 is a schematic representation of the mass fragmentation profile (mass to charge ratio) obtained by fast atom bombardment mass spectroscopy of the acetylated derivatives of the oligosaccharides obtained from endometrial PAEP. The oligosaccharides were obtained using the following protocol. Endometrial PAEP was digested with trypsin (1:50 ratio of trypsin to endometrial PAEP). The tryptic peptides were digested with N-glycanase (Boehringer Manheim Chemicals, FRG) to release the asparagine-linked oligosaccharides. Free oligosaccharides were separated from peptide fragments by reverse phase chromatography. The

oligosaccharides were derivatized by peracetylation and subjected to fragmentation analysis by Fast Atom Bombardment-Mass Spectrometry (FAB-MS) according to a previously established protocol involving A type cleavage and beta cleavage (DELL, 1994). The peracetylated oligosaccharides were dissolved in methanol and layered onto a monothioglycerol matrix on the end of a FAB probe. The sample was introduced into the atom/ion beam and the FAB spectra were recorded using a VG Analytical ZAB-2SE FPD Mass spectrometer fitted with a Cesium ion gun operated at 20-25 kvolts. Data acquisition and processing were performed using the VG Analytical Opus software. Mass fragments obtained were compared to a known library of fragment ions that have been determined for different carbohydrate sequences (DELL, 1994). The X axis is the mass to charge ratio (m/z) ranging in this figure from 1400-5000. The Y axis is a percent abundance of a particular mass fragment.

Fig. 4 is a schematic representation of the FAB-MS data obtained from peracetylated endometrial PAEP-derived N-linked oligosaccharides exactly as described in Fig. 2, except that data in the lower mass range (m/z between 200 and 1400) were recorded. The region from 900m/z to 1400 m/z is magnified 40 times.

Fig. 5 is a schematic representation of the different oligosaccharide chains that were found attached to site 1 (asparagine on amino acid 28) and site 2 (asparagine at amino acid 63). Site 3 (asparagine at amino acid 85) is not glycosylated. Abbreviations used in this figure include: HexNAc; N-acetylhexosamine (GalNAc or GlcNAc); Hex, hexose (Man or Gal); NeuAc, N-acetylneurmainic acid; and Fuc, fucose. Determination of the glycosylation site occupancy and analysis of the oligosaccharide structures was carried out using ES-MS and FAB-MS. Endometrial PAEP was digested in separate experiments with trypsin, cyanogen bromide and Staphylococcus aureus V8 protease. The glycopeptide products, obtained after digestion, were chromatographed on analytical reverse phase high performance liquid chromatography (HPLC) using 0.1% trifluoroacetic acid/acetonitrile gradients and/or analyzed by on-line microbe

liquid chromatograph (LC)-ES-MS. The glycopeptides separated by HPLC were treated with peptide N-glycosidase F to isolate the oligosaccharide chains. The released oligosaccharides were permethylated and analyzed by FAB-MS as described in Figure 3. The peptides were analyzed by ES-MS as described in Figure 3.

Fig. 6 is a schematic representation of the characterized oligosaccharides associated with endometrial PAEP. NeuAc is N-acetylneuraminic acid, Gal is galactose, GalNAc is N-acetylgalactosamine, GlcNAc is N-acetylglucosamine, Fuc is fucose, and Man is mannose. Asn is asparagine, indicating the site of covalent linkage of the oligosaccharides to endometrial PAEP.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is a discovery of the present invention that progesterone-associated endometrial protein (PAEP) (also known as placental protein 14 [PP14], pregnancy-associated endometrial protein (PEP) and human chorionic alpha-2-microglobulin) inhibits human sperm-zona pellucida binding at low concentrations (5-25 μg/ml or 1.8-8.9 X 10-7 M; see Fig. 1). This result is surprising in that PAEP is a normal endometrial glycoprotein that is expressed during the first 2-3 days of the new menstrual cycle, but then disappears and remains undetectable until five days after ovulation (JULKUNEN, 1985). Its concentration peaks between the twelfth and fourteenth postovulatory days of the cycle and then declines (SEPPALA, 1988). Interestingly, PAEP is induced to a very high level in the human decidua (10% of the total protein) following implantation of the human blastocyst (JULKUNEN, 1985). Endometrial PAEP is also secreted into the amniotic fluid, with concentrations exceeding 40 μg/ml at about 15 weeks gestational age in the human.

This evidence indicates that endometrial PAEP is a "natural contraceptive substance" that may act as a regulator of fertility in the human female. Moreover, we have been able to demonstrate that the oligosaccharides derived from endometrial PAEP block human sperm-zona pellucida binding in the HZA. Thus the carbohydrate chains appear to be responsible for the unique biological activity

of PAEP. Since we have tested many glycoproteins from diverse sources in the HZA and have not found any other inhibitors that block human sperm-zona pellucida binding at such a low concentration, it is likely that the oligosaccharides derived from endometrial PAEP are either identical to or close structural analogues of the "natural" oligosaccharide ligands that are associated with the human zona pellucida.

Although the oligosaccharides derived from endometrial PAEP are inhibitory, a higher concentration is required than necessary for the intact protein. Applicants do not wish to be bound by any theory, however it is possible that the requirement for high concentration of oligosaccharides may be due to a need for precise presentation of one or more oligosaccharide ligands to a receptor (VARKI, 1994). Such oligosaccharides may be presented in a multivalent array or in a unique combination of different oligosaccharide sequences in a specific arrangement (referred to as a "patch") that enables the native glycoprotein to manifest an inhibitory activity at significantly lower concentration than free oligosaccharides released from the native glycoprotein.

PAEP and its components are useful as contraceptive agents and are well tolerated in humans because PAEP is naturally found in the female reproductive tract. PAEP and its derivatives have the advantage of being very highly specific and more natural than the currently existing barrier type contraceptive methods that rely simply upon strong detergents that solubilize the sperm surface membranes. The disadvantage of such detergents is relatively severe irritation that often develops in susceptible individuals following the use of such contraceptive agents. The side effects associated with such a natural contraceptive as PAEP or its associated oligosaccharides are greatly reduced.

Moreover, the specificity and potency of endometrial PAEP indicate that its arrangement of one or more oligosaccharides bind with high affinity to the egg binding protein(s) located on the surface of the human sperm. Glycoconjugates that can occupy this site on the surface of human sperm are useful for assessing sperm function with regard to the binding interaction of the human sperm with the

human zona pellucida. Human sperm which fail to bind endometrial PAEP or oligosaccharides associated with endometrial PAEP indicate a specific dysfunction associated with the sperm's egg-binding protein. Such a dysfunction could be caused by the aberrant expression of PAEP-associated oligosaccharides or close structural analogues of endometrial PAEP in the seminal plasma. Any component of either the seminal plasma or substances in the female reproductive tract (e.g., anti-carbohydrate antibodies, antibodies to the egg-binding proteins, aberrantly produced oligosaccharides or glycoconjugates) that could block the interaction of endometrial PAEP with its sperm binding site could negatively affect fertility.

Similarly, antibodies that are specific for the oligosaccharide sequences of endometrial PAEP can be used to diagnose deficient or aberrant expression of carbohydrate ligand for sperm binding on the zona pellucida of an egg. This would be particularly useful to a patient undergoing *in vitro* fertilization. Once such an abnormal binding parameter is established for either the male or female counterpart of an infertile couple, that couple can be referred for direct sperm injection into the human oocyte, a procedure known as intracytoplasmic sperm injection (ICSI).

Oligosaccharides associated with endometrial PAEP may be aberrantly expressed on secretions or tissues associated with either the male or female reproductive tracts. Such individuals exhibit decreased fertility. The oligosaccharides may also be aberrantly expressed temporally, *i.e.*, expressed at a time when normal fertile humans do not express them in that tissue. Functional infertility arises due to direct inhibition of human sperm-zona pellucida binding or a decrease in the motility or non-specific acrosome reaction of affected human sperm. Sperm from infertile men have often been shown to exhibit greatly reduced motility or have much lower binding affinity for the human zona pellucida. Similarly, the mucins found in the reproductive tracts of infertile women often reduce the binding capacity of sperm in the hemizona assay. Such clinical observations may be explained by the aberrant expression of endometrial

PAEP-derived oligosaccharides on tissues or secretions of the male or female reproductive tract.

Proteins that bind to endometrial PAEP-associated oligosaccharides can be used for the diagnosis/prognosis of sperm infertility related to aberrant expression of such endometrial PAEP-associated oligosaccharides on tissues or secretions of the male or female reproductive tract. Such diagnostic methods can be used to facilitate clinical techniques used in human reproduction including intrauterine insemination, *in vitro* fertilization (IVF), gamete intrafallopian transfer (GIFT), intracytoplasmic sperm injection (ICSI), sperm cryopreservation, or methods for enhancement of human sperm capacitation. Substances that can specifically bind to the endometrial PAEP-associated oligosaccharides aberrantly expressed on the tissues or secretions of the male or female reproductive tract and inactivate them are also contemplated by the invention. Use of such substances promote the binding of the human sperm to the human zona pellucida.

Biological macromolecules which specifically bind to endometrial PAEP-associated oligosaccharides (including monoclonal and polyclonal antibodies, proteins, lectins, such as wheat germ agglutinin, concanavalin A, and Wisteria floribunda, enzymes, or active fragments thereof) can be used to bind aberrantly expressed endometrial PAEP-associated oligosaccharides or related substances on the human zona pellucida. Assessment of such binding can be employed for diagnostic/prognostic purposes to assess zona pellucida dysfunction.

The binding sites for endometrial PAEP-associated oligosaccharides on human sperm can be determined by conjugating endometrial PAEP-associated oligosaccharides to indicator molecules such as fluorophores, enzymes, radiolabels, matrices, colloidal substances, lectins, lipid carriers, biotin conjugates, proteins, polysaccharides or other substances used for tagging molecules for detection. Both primary and secondary detection methods can be employed using such reagents for detection of binding sites.

Endometrial PAEP-associated oligosaccharides are defined, for the purposes of this application, as endometrial PAEP itself, oligosaccharides derived from

endometrial PAEP, or conjugates of the endometrial PAEP-derived oligosaccharides. PAEP-associated oligosaccharides refers to those oligosaccharides, whether biologically produced or synthetically produced, which are the same as those oligosaccharides which are found on endometrial PAEP. Conjugates may contain proteins, natural or synthetic lipids, inert matrices (such as agarose beads, glass beads, polyacrylamide beads, latex beads) polysaccharides of natural or synthetic origin, polymers of amino acids, or polymers that contain groups that react with oligosaccharides at their reducing terminals. Proteins which are particularly useful are those enzymes that inhibit human sperm-zona pellucida binding. Other substances used in the art of glycobiology to couple oligosaccharides may also be used. Retention of biological activity after conjugation is desirable. Chemical mimetics or structural analogues of the oligosaccharides derived from endometrial PAEP can also be used as "endometrial PAEP-related substances" or "PAEP-associated oligosaccharides" for the purpose of this invention. Since the major oligosaccharide sequences associated with endometrial PAEP have now been determined, it is within the skill of the art to produce a recombinant form of endometrial PAEP that bears the same sequence, for example by use of a cell line which properly glycosytates human proteins such as the human kidney 293 cell line.

Endometrial PAEP-associated oligosaccharides can be modified by covalent modification with at least one molecule selected from the group consisting of sugar moieties, lipids, phosphate groups, acetyl groups, methyl groups, propyl groups, mineral acids, and polymeric molecules. Such modifications may favorably influence pharmacokinetic properties, such as half-life and tissue distribution. They may also lead to an augmented contraceptive activity level.

The oligosaccharides attached to endometrial PAEP are either similar or identical to those found on the human zona pellucida. Thus substances that can bind to endometrial PAEP-associated oligosaccharides are of both contraceptive and diagnostic/prognostic use. In the art of glycobiology, substances which are known to bind oligosaccharide sequences include monoclonal antibodies and

polyclonal antibodies. Such antibodies can be specifically designed to react with a known oligosaccharide sequence using established technologies (MAGNANI, 1987; SPITALNIK, 1987). Lectins, including wheat germ agglutinin, concanavalin A, and Wisteria floribunda, proteins and enzymes can also bind to carbohydrates and either degrade, modify or block their biological function. Once the amino acid sequence of such antibodies, proteins, enzymes or lectins are determined, amino acids in the binding site can be determined to produce biologically active fragments equivalent or more potent than the intact molecules. Substances which specifically bind to endometrial PAEP-associated oligosaccharides can be used to promote human sperm-egg binding using assisted reproduction techniques [such as intrauterine insemination, in vitro fertilization (IVF), gamete intrafallopian transfer (GIFT), intracytoplasmic sperm injection (ICSI), sperm cryopreservation or methods for enhancement of human sperm capacitation].

Endometrial PAEP-associated oligosaccharides may be administered to a patient by any appropriate route that could lead specifically to blocking human sperm-zona pellucida binding or decrease sperm motility. Such routes may be intravaginal, intrauterine, intravenous, intramuscular, subcutaneous, intradermal, oral, rectal, or inhalation. Endometrial PAEP-associated oligosaccharides or substances that bind endometrial PAEP-associated oligosaccharides could also be delivered via a contraceptive device (e.g., condom, intrauterine device (IUD), sponge or diaphragm) or as an admixture with a pharmaceutically acceptable carrier that would come into contact with human sperm during or after coitus.

The present invention is based on the discovery that endometrial PAEP and endometrial PAEP-associated oligosaccharides inhibit human sperm-zona pellucida binding. Useful forms of endometrial PAEP for the purpose of this invention include natural and recombinant forms of endometrial PAEP as well as derivatives, oligosaccharides or fragments of endometrial PAEP (including chemically synthesized forms, mimetics or analogues of such derivatives and fragments). Such forms of endometrial PAEP-associated oligosaccharides (1) retain ability to

block human sperm-zona pellucida binding, and (2) can be used for diagnosis/prognosis of human sperm-zona pellucida binding defects. One particularly useful derivative of endometrial PAEP-associated oligosaccharides contains half sulfate esters in place of sialic acid residues on the oligosaccharides. Half sulfate esters having a single negative charge have been shown to substitute for sialic acid on oligosaccharides with complete retention of biological activity (VARKI, 1994).

EXAMPLES

Example 1

This example demonstrates the inhibitory effect of PAEP on human sperm-egg binding.

The inhibitory effect of endometrial PAEP on human sperm-zona pellucida binding activity is shown in Fig. 1. These data indicate that sperm binding was almost completely eliminated at very low concentrations of endometrial PAEP. Since the molecular weight of endometrial PAEP is 28 kDa, this inhibition of human sperm-zona pellucida binding is manifested at a molar concentration not exceeding 1.8×10^{-6} M. This outcome itself is achieved without any noticeable effect on human sperm motility at such concentrations (Table 1). Although it is possible that at higher, non-physiological, concentrations endometrial PAEP will decrease sperm motility, such an effect would aid its overall contraceptive activity.

Example 2

This example demonstrates the inhibitory effect of PAEP oligosaccharides on sperm-egg binding.

The human egg is coated with a thick extracellular matrix known as the zona pellucida. This extracellular matrix is the initial site of contact for human sperm binding during normal fertilization. Therefore specific agents that can block the initial binding of human sperm to the zona pellucida would have a primary contraceptive effect. To assay the contraceptive activity of different substances, we have employed the hemizona assay system (BURKMAN, 1988). The HZA involves the microbisection of the human egg, resulting in the release of the egg

cell contents and the generation of two equally matched hemispheres of zona pellucida (hemizonae). By comparing the binding of fertile sperm in the presence and absence of a test substance, it is possible to quantitate the contraceptive effect of the test substance using this internally controlled assay system.

Studies in other mammalian systems suggested that human sperm-zona pellucida binding might be contingent upon a carbohydrate-dependent adhesion system. Therefore we tested whether endometrial PAEP-derived oligosaccharides could inhibit in the HZA. At a concentration of 10 μg/ml, there was on average 22% inhibition of binding without loss of motility (Table 2). At 25 μg/ml, there was a 50% decrease in human sperm binding to the human zona pellucida. At 50 μg/ml there was nearly complete inhibition of binding, but a decrease in the sperm motility was also observed. These results suggest a relatively potent inhibitory effect of endometrial PAEP-derived oligosaccharides on human sperm-zona pellucida binding. This result is consistent with the inhibition manifested by intact endometrial PAEP.

Example 3

This example demonstrates the chemical identity of PAEP carbohydrate.

Endometrial PAEP is a glycoprotein consisting of 17.5% carbohydrate (BOHN, 1982). To determine the structure of the biologically active oligosaccharides, chemical analysis of these components and their linkage site to the endometrial PAEP protein backbone was performed. The natural form of endometrial PAEP consists of 162 amino acids (JULKUNEN, 1988). There are three sites for asparagine-linked (N-linked oligosaccharides on the protein (at amino acids 28, 63 and 85 from the N-terminal end of the protein) (Fig. 2).

Endometrial PAEP was trypsin-digested to obtain peptides and glycopeptides that were separated by reverse phase chromatography and analyzed by electrospray mass spectrometry (ES-MS) as previously described (DELL, 1994). Comparison of the fragmentation pattern with the known protein structure indicated that the asparagine at the 85th amino acid was not glycosylated. However, the first site (asparagine at position 28) was found to always be

glycosylated. The second site (asparagine at position 63) was glycosylated on a subset of the glycoforms of endometrial PAEP. A method known as fast atom bombardment-mass spectrometry (FAB-MS) (DELL, 1994) was employed to determine the sequence of the asparagine-linked oligosaccharides derived from endometrial PAEP. The detection of several high mass fragment ions (at m/z 1557, 1770, 1959, 2164, 2368, 2624, 2798, 2986, 3189, 3626, 4000, and 4878) during this analysis indicates that the oligosaccharides derived from endometrial PAEP are very heterogeneous in their structure (Fig. 3).

The identification of the fragment ions is based upon the known m/z values obtained from an established library for carbohydrate sequences (DELL, 1994). Analysis conducted in this upper mass regions (m/z exceeding 1400) indicated that the majority of the intact N-linked endometrial PAEP-derived glycans are in the mass range expected for biantennary or hybrid type structures while a minority are in the mass range for tri- and tetra-antennary structures and/or bi- and triantennary structures with repeating N-acetyllactosamine type sequences.

Analysis of the fragments obtained from m/z below 1400 was also carried out (Fig. 4). The notable features of this fragmentation data obtained in the m/z below 1400 include: (1) the existence of antennae having two N-acetylhexosamine (HexNAc) residues which can be unsubstituted, fucosylated, or sialylated but not both sialylated and fucosylated; linkage analysis experiments before and after treatment with exoglycosidases indicated that the antennae contain GalNAcβ1-4GlcNAcβ1 which can be sialylated on the 6 position of the GalNAc or fucosylated on the 3-position of the GlcNAc; and (2) in addition to the antennae described in (1), there are antennae comprised of the normal mammalian building block viz N-acetyllactosamine (Galβ1-4GlcNAcβ1-) which occurs in up to three repeats. These antennae can be unsubstituted, sialylated at the 3-, or 6-position of the Gal, or fucosylated at the 3-position of the GlcNAc. There is the possibility for a small amount of oligosaccharide bearing the sialyl Lewis^x determinant on its antennae (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-R). The Lewis^x epitope

(Galβ1-4[Fucα1-3]GlcNAcβ1-R) may be expressed on the antennae of a substantial subset of the glycans.

Endometrial PAEP was digested in separate experiments with trypsin, cyanogen bromide and Staphylococcus aureus V8 protease to obtain peptides and glycopeptides that were separated by reverse phase high performance liquid chromatography (HPLC) using 0.1% trifluoroacetic acid/acetonitrile gradients and analyzed by on-line liquid chromatograph (LC) and direct injection electrospray mass spectrometry (E-MS) as previously described (DELL, 1994). Comparison of the masses obtained with the known protein structure allowed identification of the putative glycosylation positions in the glycoprotein and determination of which are occupied and with which oligosaccharide structures. Asparagine 85 was found not to be glycosylated. However, the first potential glycosylation site (asparagine at position 28) and the second site (asparagine 63) were found to be glycosylated both from the ES-MS mass data and by the FAB-NS analysis of permethylated oligosaccharides released by peptide N-glycosidase F from the purified glycopeptides.

Based upon our ES-MS and FAB-MS analysis, a library of different oligosaccharide chains attached to the first and second N-glycosylation site of endometrial PAEP was determined (Fig. 5). The structures of the major endometrial PAEP glycans that we have fully characterized can be divided into the categories as shown in Fig. 6. Structures falling in group 1 are unusual biantennary type oligosaccharides with GalNAcβ1-4GlcNAc linked via β1-2 to mannose and N-acetyllactosamine (Galβ1-4GlcNAc) linked to mannose on the other arm. Up to two moles of fucose can be linked to this core structure. Known fucose linkages in this case are Fucα1-3GlcNAc (in the outer antenna) or Fucα1-6GlcNAc at the GlcNAc proximal to the Asn. N-acetylneuraminic acid may or may not be attached via α2-3 or α2-6 linkages to either terminal GalNAc or Gal. Structures falling in group 2 are similar to group 1, except that both antennae are GalNAcβ1-4GlcNAc sequences. Structures in group 3 are similar to group 1 except that fucose is attached only to the GlcNAc proximal to the Asn

linkage site and N-acetylneuraminic acid is attached via α2-3 or α2-6 linkages to both GalNAc and Gal at terminal positions of the core structure. Structures in group 4 and 5 fall into the category known as hybrid type oligosaccharides, with mannosyl residues in the antennae and a sialylated N-acetyllactosamine or sialylated GalNAcβ1-4GlcNAc sequence attached to the other antennae. Sequences falling in group 6 are relatively common and are known as high mannose type structures.

Our analysis indicates that the majority of the PAEP-associated glycans have determinants that have been previously identified on pituitary glycohormones (GREEN 1986). In particular the GalNAcβ1-4GlcNAc terminal sequence has been associated with hormones that have a specific tripeptide sequence Proline-(any amino acid)-Arginine/Lysine (Pro-X-Arg/Lys) (DHARMESH 1993). This tripeptide is a recognition sequence for a β1,4- N-acetylgalactosaminyltransferase that is necessary for the synthesis of terminal β1,4-linked GalNAc sequence. The sialylated GalNAcβ1-4GlcNAc sequence has been found in human lutropin (GREEN 1986) and Bowes melanoma tissue plasminogen activator (CHAN 1991). The terminal sequence (GalNAcβ1-4[Fucα1-3]GlcNAc-R) has been found as a major structure in lower animals (e.g. schistosomes, filarial worms and egg jelly coats of Axolotyl maculatum) (SRIVATSAN 1992; STRECKER 1994). In humans it has been identified in two glycoproteins, namely human urokinase (BERGWERFF, 1992) and human recombinant protein C expressed by human kidney 293 cells (YAN, 1993). The presence of substantial amounts of GalNAcβ1-4GlcNAc sequences on endometrial PAEP is rare for any glycoprotein and may reflect its unusual ability to inhibit human sperm-zona pellucida binding.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Clark, Gary F.
Oehninger, Sergio
Patankar, Manish S.
Seppala, Markku T.
Koistinen, Ritta
Dell, Anne
Morris, Howard R.

(ii) TITLE OF INVENTION: Glycoconjugate Inhibitors of Human Sperm-Egg Binding

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Banner & Allegretti, Ltd.
(B) STREET: 1001 G Street, N.W.
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(F) ZIP: 20001-4597

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kagan, Sarah A.
(B) REGISTRATION NUMBER: 32,141
(C) REFERENCE/DOCKET NUMBER: 0570.54658

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 202-508-9100
(B) TELEFAX: 202-508-9299

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 819 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 45..587

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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 85                  90                  95                 100

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Lys Ile Asn Tyr Thr Val Ala Asn Glu Ala Thr Leu Leu Asp Thr Asp
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(2) INFORMATION FOR SEQ ID NO:2:

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(A) LENGTH: 180 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CLAIMS

1. A method for inhibiting human sperm-zona pellucida binding, comprising:

administering a composition comprising endometrial PAEP-associated oligosaccharides to a female, in an amount effective to inhibit human sperm-zona pellucida binding.

2. The method of claim 1 wherein said composition comprises endometrial PAEP.

3. The method of claim 1 wherein said composition comprises oligosaccharides derived from endometrial PAEP which are conjugated to a polymer.

4. The method of claim 3 wherein the polymer is a polypeptide.

5. The method of claim 3 wherein the polymer is a polysaccharide.

6. A method for inhibiting human sperm-zona pellucida binding, comprising:

administering to a female a biological macromolecule which specifically binds to endometrial PAEP.

7. The method of claim 6 wherein the biological macromolecule is an antibody.

8. The method of claim 6 wherein the biological macromolecule is a lectin.

9. A method for assessing human sperm dysfunction, comprising:

determining the number of binding sites for endometrial PAEP on a sample of human sperm, wherein said number of binding sites correlates with ability of said sperm to bind to human zona pellucida.

10. The method of claim 9 wherein said step of determining employs a competitive binding assay.

11. The method of claim 9 wherein said number of binding sites are determined using antibodies specific for endometrial PAEP.

12. The method of claim 9 wherein said number of binding sites are determined using lectins specific for oligosaccharides on endometrial PAEP.

13. The method of claim 9 wherein said step of determining employs endometrial PAEP-associated oligosaccharides conjugated to an indicator molecule.

14. The method of claim 9 wherein said step of determining employs endometrial PAEP-associated oligosaccharides coupled to an inert matrix.

15. A method for assessing a cause of infertility, comprising:

detecting aberrant expression of oligosaccharides normally found on endometrial PAEP in secretions or tissues of a male or female reproductive tract wherein said oligosaccharides are not expressed in said secretions or tissues of fertile humans.

16. The method of claim 15 wherein biological macromolecules which specifically bind to endometrial PAEP-associated oligosaccharides are employed in said step of detecting.

17. The method of claim 16 wherein the biological macromolecule is an antibody.

18. The method of claim 16 wherein the biological macromolecule is a lectin.

19. A method of treating an individual aberrantly expressing endometrial PAEP-associated oligosaccharides, comprising:

administering a biological macromolecule which specifically binds endometrial-associated oligosaccharides to an individual aberrantly expressing endometrial PAEP-associated oligosaccharides, wherein said biological macromolecule is administered in an amount sufficient to promote human sperm binding to human zona pellucida.

20. The method of claim 19 wherein the biological macromolecule is an antibody.

21. The method of claim 19 wherein the biological macromolecule is a lectin.

22. The method of claim 19 wherein said individual is undergoing an assisted reproduction technique.

23. A contraceptive composition comprising endometrial PAEP-associated oligosaccharides and a pharmaceutically acceptable carrier for vaginal, or intrauterine administration.

24. The composition of claim 23 further comprising a second contraceptive substance.

25. The method of claim 23 wherein the composition is free of protein.

26. The method of claim 23 wherein the composition is free of endometrial PAEP.

27. A contraceptive device impregnated with a composition comprising endometrial PAEP-associated oligosaccharides and a pharmaceutically acceptable carrier, wherein said device is selected from the group consisting of: condoms, intrauterine devices, diaphragms, and sponges.

28. A method of screening for contraceptive substances, comprising:

contacting a test substance with endometrial PAEP-associated oligosaccharides; and

determining those test substances which bind to endometrial PAEP-associated oligosaccharides.

29. The method of claim 28 wherein the endometrial PAEP-associated oligosaccharides are coupled to an inert matrix.

30. A method of screening for autoantibodies which contribute to infertility in a body sample, comprising:

contacting a body sample which comprises antibodies with endometrial PAEP-associated oligosaccharides; and

determining the presence of antibodies in the body sample which specifically bind to endometrial PAEP-associated oligosaccharides.

31. The method of claim 30 wherein the endometrial PAEP-associated oligosaccharides are coupled to an inert matrix.

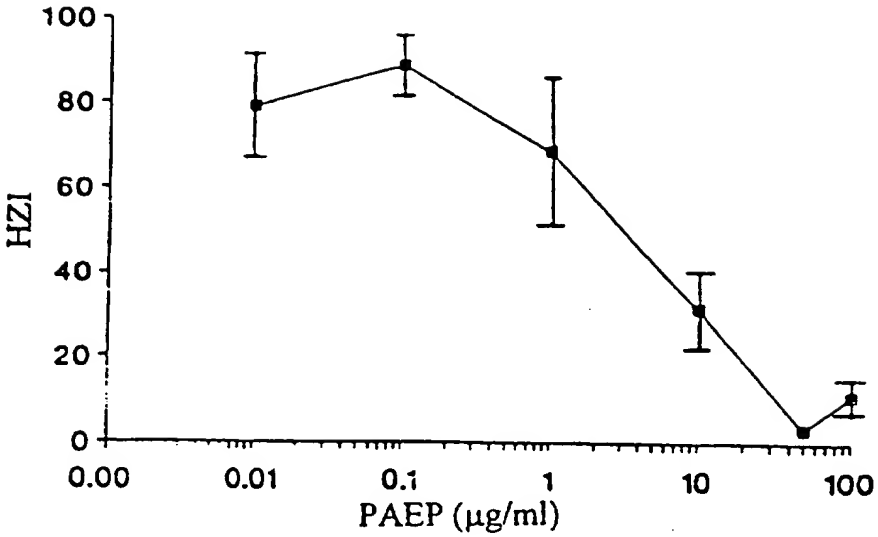
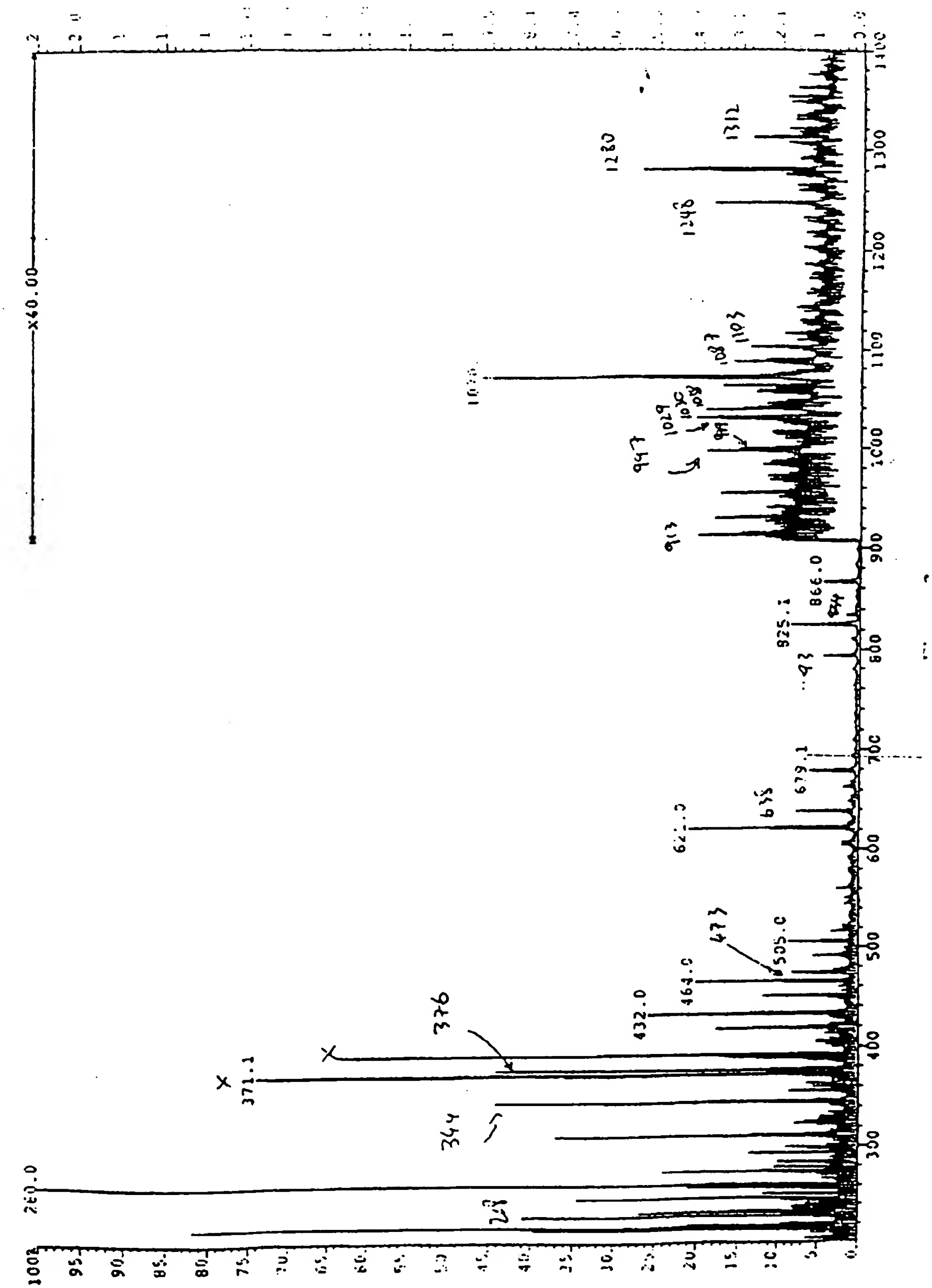


Figure 1

Figure 2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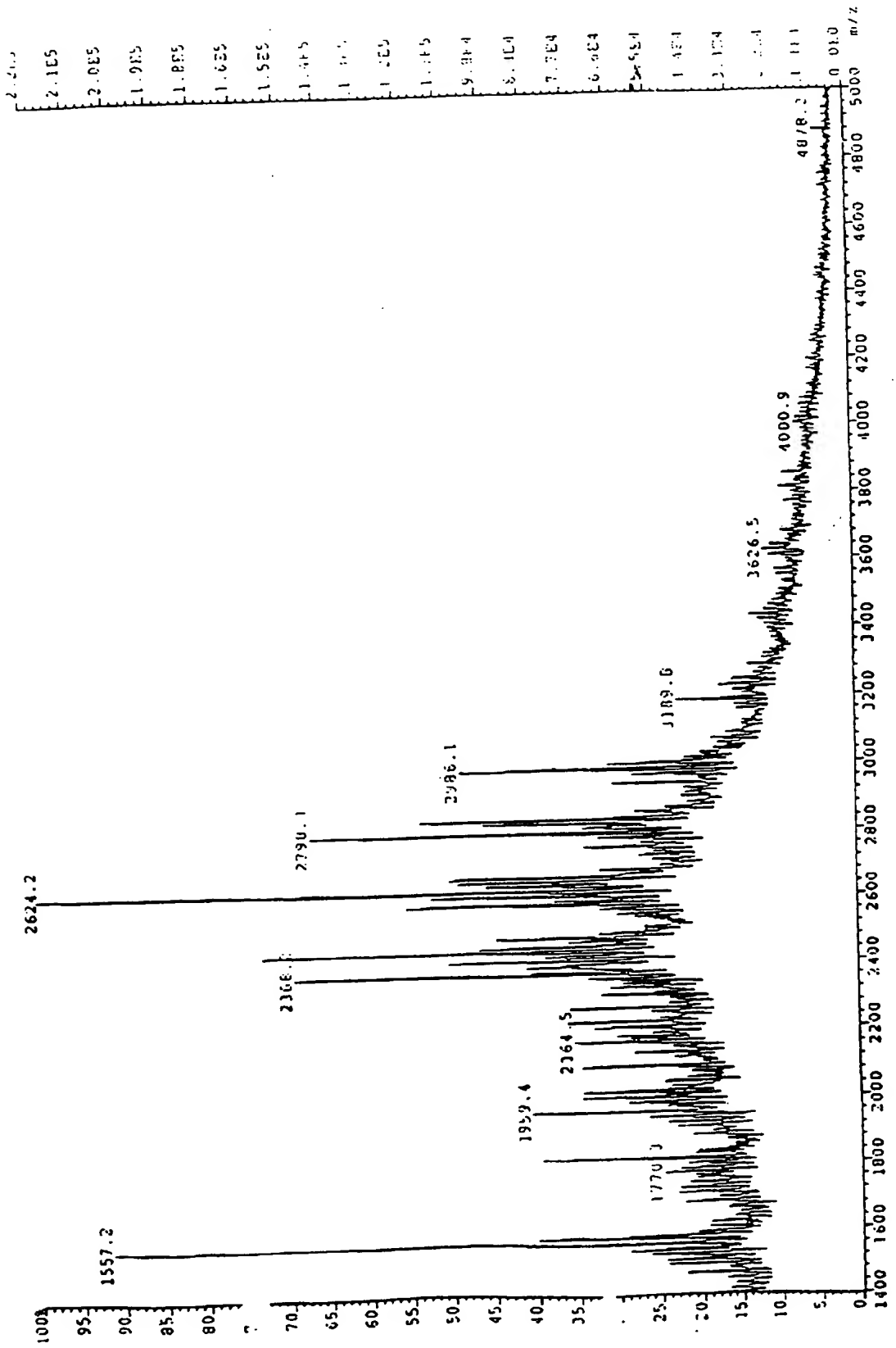
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Glycosylation Site 1 (Residue 28)

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$NeuAc_1Hex_4HexNAc_3$

$NeuAc_1Hex_3HexNAc_4$

$NeuAc_1Hex_5HexNAc_3$

$NeuAc_1Hex_4HexNAc_4$

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$NeuAc_1Fuc_1Hex_5HexNAc_4$

$NeuAc_1Fuc_1Hex_4HexNAc_5$

$NeuAc_1Hex_5HexNAc_5$

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Glycosylation Site 2 (Residue 63)

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$NeuAc_1Fuc_1Hex_3HexNAc_6$

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$NeuAc_1Fuc_2Hex_3HexNAc_6$

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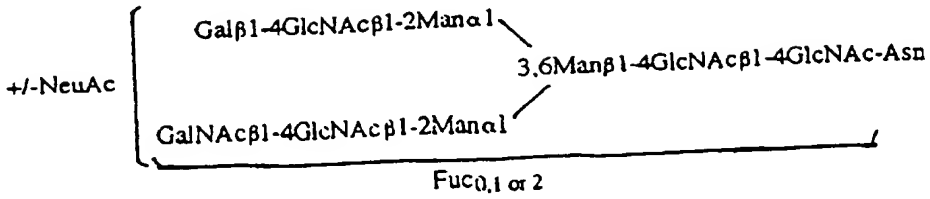
Figure 5



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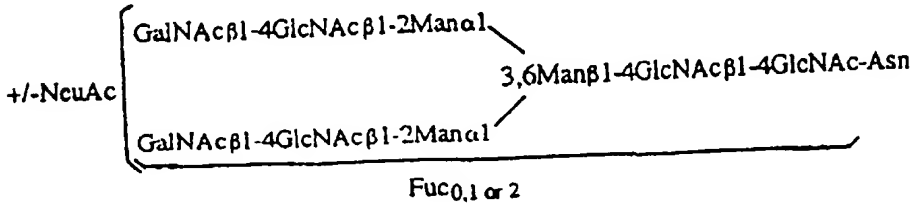

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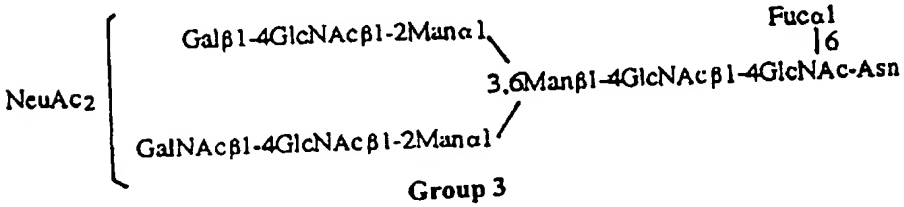

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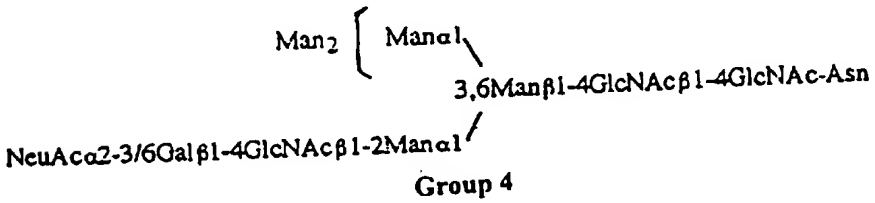

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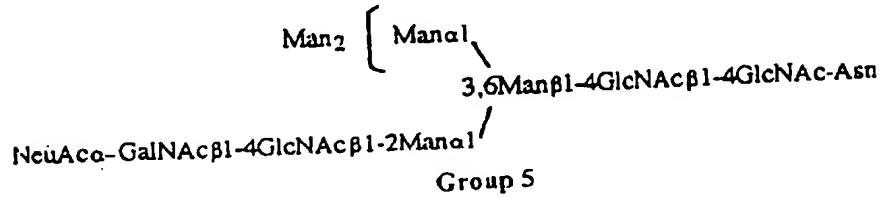
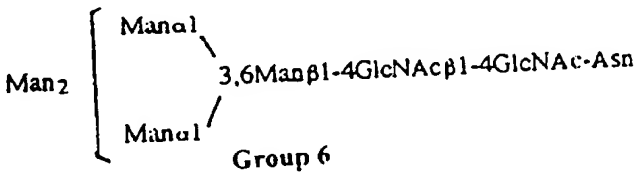


Group 5



Group 6

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02695

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	DELL et al. Structural Analysis of the Oligosaccharides Derived from Glycodelin, a Human Glycoprotein with Potent Immunosuppressive and Contraceptive Activities. The Journal of Biological Chemistry. 13 October 1995, Vol. 270, No. 41, pages 24116-24126, see entire document.	1-31
Y	OEHNINGER et al. Factors affecting fertilization: endometrial placental protein 14 reduces the capacity of human spermatozoa to bind to the human zona pellucida. Fertility and Sterility. February 1995, Vol. 63, No. 2, pages 377-383, see entire document.	1, 2, 6-11, 13, 14, 23, 24, 26, 27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 JUNE 1996	Date of mailing of the international search report 11 JUL 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RON SCHWADRON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02695

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02695

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 31/70, 31/75, 38/00, 38/16, 38/17, 39/00, 39/395; C07K 14/00, 14/435, 16/00, 16/18, 16/44; G01N 33/53, 33/564, 33/566, 33/577

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/137.1, 145.1, 152.1, 172.1; 435/7.1, 7.2, 7.92; 436/501, 510, 536, 906; 514/2, 21, 23; 530/300, 350 , 370, 387.5, 388.2,, 388.24, 389.1, 395, 396

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

424/137.1, 145.1, 152.1, 172.1; 435/7.1, 7.2, 7.92; 436/501, 510, 536, 906; 514/2, 21, 23; 530/300, 350 , 370, 387.5, 388.2,, 388.24, 389.1, 395, 396

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, BIOSIS, EMBASE, DERWENT WPI, CHEM AB, APS search terms: author names, paep,, pp14, pep, placental protein 14, pregnancy-associated endometrial protein, human chorionic alpha-2-microglobulin, endometrial, carbohydrate, sperm, zona pellucida, oligosaccharides, lectin, antibody, contraceptive, pregnancy, fertility

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

I. Claims 1-5, 23-31 are drawn to compositons of PAEP associated oligosaccharides and methods that use said compositions.

II. Claims 6-22 are drawn to methods using biological molecules which bind PAEP.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.2, because they lack the same or corresponding special technical features for the following reasons:
Group I is drawn to compositons of PAEP associated oligosaccharides and methods that use said compositions, while group II is drawn to methods that do not use PAEP associated oligosaccharides (eg. they use antibodies or lectins).